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Review Article

A Symphony of Regulations Centered on *p63* to Control Development of Ectoderm-Derived Structures

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The p53-related transcription factor p63 is critically important for basic cellular functions during development of the ectoderm and derived structure and tissues, including skin, limb, palate, and hair. On the one side, p63 is required to sustain the proliferation of keratinocyte progenitors, while on the other side it is required for cell stratification, commitment to differentiate, cell adhesion, and epithelial-mesenchymal signaling. Molecules that are components or regulators of the p63 pathway(s) are rapidly being identified, and it comes with no surprise that alterations in the p63 pathway lead to congenital conditions in which the skin and other ectoderm-derived structures are affected. In this paper, we summarize the current knowledge of the molecular and cellular regulations centered on p63, derived from the comprehension of p63-linked human diseases and the corresponding animal models, as well as from cellular models and high-throughput molecular approaches. We point out common themes and features, that allow to speculate on the possible role of p63 downstream events and their potential exploitation in future attempts to correct the congenital defect in preclinical studies.

1. The *p63* Gene: Structure and Function

The *TP63* gene codes a transcription factor homologous to the p53 tumour suppressor protein and is translated into six protein isoforms: the TransActivating (TA) isoforms are more closely resembling p53, while the Delta-N (ΔN) isoforms are devoid of the TransActivation (TA) domain 1 (TA1). Although the TA isoforms were initially thought to be the only ones to possess transcriptional regulatory functions, it has been well established that the ΔN isoforms are also able to activate transcription of a distinct set of target genes via a second TA domain (TA2) [1]. Initially, TA and ΔN isoforms with three alternative carboxyl termini (α, β, and γ), generated by alternative splicing, were identified. However, like for p73, additional p63 isoforms (δ and ε) have been recently described [2].

The p63 protein contains a TA domain, a DNA-Binding Domain (DBD) and an Oligomerization Domain (OD). Both the TA and ΔN alpha-isoforms also contain a Sterile Alpha Motif (SAM) domain, which is absent in p53 [3]. SAM domains are protein-protein interaction modules found in developmentally relevant proteins [4]. Recent studies have identified a Transcriptional Inhibitory (TI) domain located between the SAM domain and the C-terminus of p63α; this domain is believed to be responsible for the lower transactivation ability of TAp63α compared to the -β and the -γ isoforms [5].

p63 is highly conserved among a wide range of organisms. Interestingly, in the genome of *Danio rerio* (zebrafish) the mammalian type TA coding sequence is missing; therefore, only the ΔN isoforms are present [6]. Both mice and zebrafishes lacking *p63* expression fail to develop a normal epidermis and show malformations of appendages and other structures that require epithelial-mesenchymal interactions during development [7–9].

2. Human Diseases Linked to *p63*

The *p63* locus is consistently transcribed by basal stem cells of stratified epithelia, by myoepithelial cells of the breast

and salivary glands, and by the proliferative compartment of the gastric mucosa [10, 11]. The expression of the ΔNp63 and TAp63 classes of isoforms is quite distinct and changes dynamically during development and maturation of the ectoderm (towards the epidermis) and derived structures (hair follicles, limbs, glands) [11, 12]. Unlike p53, p63 does not act as a classical tumour-suppressor, although its involvement in tumor progression is being increasingly recognized [13, 14]. p63 plays a prominent role in the control of epithelial stem cell functions and in the differentiation and stratification of ectoderm-derived tissue during embryonic development.

A wide spectrum of autosomal-dominant hereditary diseases is associated to mutations of the *p63* gene, in humans. A common feature of these disorders is the appearance of Ectodermal Dysplasia (ED), a phenotypic group that comprises abnormal maturation and stratification of the skin and abnormal development of hairs, teeth, nails, esocrine glands, and cornea. The other two consistent features of *p63*-linked disorders are Cleft Lip/Palate (CL/P) and ectrodactyly, also known as Split Hand/Foot Malformation (SHFM). Ectrodactyly is a limb developmental malformation characterized by a medial cleft, missing central digits and often fusion of the remaining ones [15].

Human autosomal dominant syndromes associated with heterozygous *p63* mutations are Ectrodactyly-Ectoder-mal dysplasia-Cleft lip/palate syndrome (EEC, MIM 106260), Limb-Mammary Syndrome (LMS, MIM 603543), Ankyloblepharon Ectodermal dysplasia Clefting (AEC, MIM 106260), and nonsyndromic Split-Hand/split-Foot Malformation-type IV (SHFM-IV) (MIM 605299) [16–20]. EEC is the most prominent congenital disorder and is characterized by the three phenotypes: ED, ectrodactyly, and CL/P. EEC is almost invariably caused by heterozygous mutations in the DBD of p63 [21, 22]. Nonetheless, *p63* mutations can explain only a minority of patients with isolated or nonsyndromic ectrodactyly (about 10%) or cleft lip/palate (about 0.1%).

In addition to SHFM-IV, associated to *p63* mutations, ectrodactyly appears as an isolated nonsyndromic disorder linked to mutations or chromosomal anomalies in distinct loci [20, 23, 24]. Of these, gene mutations have been identified only for SHFM-VI (MIM 225300, the only autosomal recessive form) and for Ectodermal Dysplasia-Ectrodactyly-Macular Dystrophy syndrome (EEM, MIM 225280). In SHFM-VI, homozygous mutations of the *WNT10B* gene have been found [25]. In EEM patients, mutations in the *P-cadherin* (or *cadherin-3*, or *CDH3*) gene have been found [26, 27]; this gene codes for an adhesion molecule of the cadherin class, expressed by the AER cells of the embryonic limb buds. However, the targeted deletion of *P-cadherin* in mice does not cause limb defects [28]. It is not known, at the moment, if a regulatory link exists between p63 and these two SHFM disease genes.

SHFM-I (MIM 183600) is linked to deletions, inversions, and rearrangements affecting chromosome 7q21 [29–31]. The smallest region of overlapping deletions encompasses several genes: *DYNC1l1, SLC25A13, DSS1, DLX5*, and *DLX6*; of these only *DLX5* and *DLX6* have been shown to be specifically expressed in the AER of the developing limb buds [32–34]. Importantly, the murine models of *Dlx5;Dlx6* inactivation establish that their misregulation is etiological to SHFM-I [33, 34], and *Dlx5* and *Dlx6* are now recognized as transcriptional targets of p63 [35, 36].

SHFM-III (MIM 600095) is associated with complex duplications/rearrangements around the *DACTYLIN* (*FBXW4*) gene, on chromosome 10q [37]. The genomic lesion involves the *DACTYLIN, LBX1*, and *βTRCP* genes, but none of these genes is directly disrupted and no point mutation has been reported. Interestingly, *FGF8* and *NFkB2*, two genes implicated in limb development, are located in the proximity of the rearrangement breakpoints [38–41].

Finally, SHFM-V (MIM 606708) is associated to deletions encompassing the *HOXD* gene cluster, near *DLX1* and *DLX2* [42–44]. The X-linked SHFM-II form (MIM 313350) has been mapped to chromosome Xq26.3 [45], but no disease gene has yet been identified.

Diseases grouped within the same phenotypic class are likely to be caused by genetic abnormalities or misregulation of functionally related genes, or genes that are component of a regulatory network [46–48]. Elucidation of functional interactions among genes within the p63 network, their encoded proteins, and regulatory elements controlling their expression is bound to provide new candidate genes for genetic disorders linked to p63.

3. Genotype-Phenotype Correlations

In the EEC spectrum syndromes, the position and type of p63 mutation (frameshift, missense, deletion) correlate with the observed phenotype. *p63* mutations causing EEC are usually not found in AEC, LMS, and SHFM [16–19]. The vast majority of EEC mutations are missense mutations in the DBD, generating aminoacid substitutions in the residues predicted to contact DNA. In these cases, all p63 isoforms are affected by these mutations. DBD mutants usually act as dominant-negative effectors and render the WT protein unable to bind DNA [21], explaining the dominant transmission of EEC. Mutations in exons 13 and 14, affecting only the *α* isoform of p63, have been found associated to all four syndromes.

SHFM-IV results from mutations either in the DBD or in the C-terminal domain of p63*α*, whereas the AEC syndrome is exclusively associated with heterozygous missense mutations in the SAM domain. The *α* tail of p63 contains a sumoylation site, inactivated by *p63* mutations found in SHFM-IV (E639X). Sumoylation can modulate p63 half-life [1], and naturally occurring mutated p63 proteins often display altered stability, suggesting that the final effect of the mutations could be the persistence of the mutated protein and consequent misexpression of p63 targets.

Finally, for a large set of disorders with features of ED and resembling *p63*-caused syndromes (referred to as p63-linked phenotype spectrum), the molecular lesion is unknown [49].

Attempts to unequivocally establish the role of p63 in human ectodermal syndromes are hampered by the fact that this protein exists in multiple isoforms with different, often contradictory, biological activities; moreover, some of the

mutations are isoform-specific (affecting only the α isoform) while others affect all isoforms.

Therefore, the mode of action of *p63* mutations (haploinsufficiency? dominant-negative?) is still debated. For the same reason, the possibility to use wild-type p63 for pharmacological purposes or for gene delivery appears unrealistic.

4. Animal Models of *p63*-Linked Diseases

Much of our current knowledge on the role of disease genes for ectoderm development and limb morphogenesis has been gathered via the generation of animal models of the EEC, AEC, and SHFM, and the analysis of their phenotypes at cellular and developmental level. Mice null for *p63* have been generated by two groups independently [50, 51]; at birth these mice show severe defects affecting their skin, limb and craniofacial skeleton, teeth, hair, and mammary glands. Specifically, the skin appears thin, mostly single-layered and translucent, unable to prevent water loss. The hindlimbs (HL) fail to form altogether, while the forelimbs (FL) are severely truncated and lack most of their distal skeletal elements. The altered phenotypes observed in these mutant mice are a direct consequence of altered cellular properties affecting the same tissues and organs as in human EEC, thus these mice have been considered as models of human EEC [21, 50–52].

However, there are some caveats intrinsic to the *p63* null mice as model of EEC. First, in the *p63* null model named *Brdm2* [50], gene inactivation was achieved by insertional mutagenesis, which however resulted in a partial gene duplication. Although the p63 protein is undetectable in these mice, their skin shows patches of stratified epithelium that appear to reexpress *p63*, and it is currently unclear whether these are due to aberrant transcription of the mutated *p63* gene or represent spontaneous phenotypic reversal [53]. Second, and this is the major concern, loss of *p63* in the germline is not synonymous of the presence of one mutated allele, the situation commonly observed in EEC patients. While in the null animals the p63 protein is missing altogether (i.e., both the TA and ΔN isoforms), in EEC, AEC, LMS, and SHFM-IV patients the mutated p63 protein coexists with half of the normal dose of wild-type p63. Hence, the *p63* null mice might not accurately recapitulate the molecular pathogenesis of these syndromes.

To circumvent this problem, the group of Dr. A. Mills (CSHL, USA) has generated a new line of mice bearing the *R279H* mutation in *p63* (a mutation found in EEC patients) in their germline. Homozygous embryos and newborn animals show a global phenotype similar, but not identical, to that of *p63* null animals [35], consisting in the absence of the HL, severely truncated FL, a thin translucent skin and craniofacial and palatal defects. The HL defects in both the *p63* null- and the *p63-R279H* homozygous embryos are evident as early as E9.5, accompanied with loss of AER stratification [35, 50, 51]. Interestingly, heterozygous *p63-R279H* mutant embryos and newborns show a poorly penetrant EEC phenotype characterized by mild skin hyperplasia, cleft palate, and ectrodactyly. In particular, the skin of these animals shows patches of hyperplasia, in which *Irf6* and *IKKα* expression is markedly reduced, while p63 nuclear expression is increased. The same can be observed in skin of AEC patients [54, 55]. Thus, the *p63-R279H* heterozygous mice display a global phenotype that resembles human EEC more closely than the phenotype of *p63* null mice.

Mice have been generated in which ΔNp63 is down-regulated in the developing epidermis, as a model for AEC [56]. In fact, the AEC-mutated variants of Δ*Np63* have been shown to exert a dominant-negative action on the wild-type protein. Reduced ΔNp63 expression in the mouse epidermis causes severe skin erosion consequent to suprabasal cell proliferation, retarded terminal differentiation and basement membrane abnormalities [56], a condition that closely resembles the AEC phenotype.

Mouse models for SHFM-I have been generated by the combined deletion of *Dlx5* and *Dlx6* [33, 34]; in these animals ectrodactyly is observed at the HL, the AER is induced normally, however, by E10.5 the expression of *FGF8* and other markers declines in the central sector of the limb bud, accompanied with a loss of stratification in the same region [31, 32] (G.M. and LoIacono, unpublished).

The *Dactylaplasia (Dac)* mutant mouse strain has been proposed as a model of SHFM-III [57, 58]. The AER of *Dac* heterozygous embryos shows reduced *FGF8* expression and defective cell layering [59]. However, the role of *Dactylin* as the disease gene in SHFM-III is currently doubted, since the rearrangements/duplications around the *Dactylin* locus do not disrupt or interrupt the gene, and since *Dactylin* is ubiquitously expressed in mouse tissues.

Finally, zebrafish *p63* morphants have been reported [6, 60]. Interestingly, they share some phenotypic features with human EEC patients and might be used for developmental studies. In this model, ΔNp63 expression is tightly regulated by Smad4/5-mediated Bone Morphogenetic Proteins (BMP) signaling, and it acts as a transcriptional repressor that blocks anterior neural specification and conversely promotes early steps of epidermal specification. Forced expression of ΔNp63γ blocks neural development even in the absence of BMP signal [6]. We should point out, however, that the skin and fins of fishes are not fully comparable to the mammalian limbs [61], and therefore the information gained from the fish model might not fully translate to the mammalian system.

5. The Role of *p63* for Keratinocyte Proliferation and Differentiation

The best experimentally characterized function of p63 is to maintain the proliferative potential of epidermal progenitor cells [62, 63]. In addition to this, p63 also impacts on epidermal stratification and keratinocyte differentiation. Specifically, ΔNp63 is required for the initial commitment of keratinocyte progenitors towards differentiation [10, 62], although for differentiation to proceed ΔNp63 needs to be subsequently eliminated [64, 65]. The depletion of ΔNp63 occurs mainly via proteasome-mediated degradation [66–68], which in turn is controlled by several proteins some of which are transcriptional targets of ΔNp63 [55, 69, 70]. In addition, the expression of a p63-specific microRNA

(miR203) is also important to induce p63 downregulation during terminal differentiation [71].

p63 regulates transcription via binding to p63-Response Elements (RE), which in some cases also function as p53 RE. Indeed, many of the p53 RE involved in DNA damage-induced cell cycle arrest or apoptosis are constitutively occupied by ΔNp63 in proliferating keratinocytes [72–74] (A.C., unpublished results). Regulation of binding to these elements and changes in the transcriptional capabilities of ΔNp63 are thought to play a role in promoting the exit from the cell cycle of keratinocytes at the onset of their differentiation.

ΔNp63 can control distinct transcriptional networks depending on the state of maturation of keratinocyte precursors, which in turn is dependent on a variety of extracellular stimuli. In proliferating keratinocytes of the basal layers, ΔNp63 can control the expression of basal layer keratins (*K5*, *K14*), of molecules required for the formation of the epidermal barrier, such as *Alox12* [75], and inhibit proliferation-induced activation of cell cycle arrest genes by competing with p53 for the same responsive elements. Following a differentiation stimulus, ΔNp63 can change its transcriptional activity, detach from the promoter of cell cycle arrest genes (e.g., *14-3-3 sigma* and *p21waf1*), activate genes required for cell cycle exit (*IKKa* and *IRF6*), and reorganize the transcription of adhesion molecules to allow keratinocytes to leave the basal layer and stratify (see Figure 1). Differences in the temporal expression, isoform combination, biochemical properties, and transcription activity of p63 protein(s) can have profound impact on the set of genes being transcribed, at a given time in a given cell. This notion implies that during development, p63 protein(s) exert distinct roles, in a time and region-specific manner, an issue that will be resolved only with the generation of isoform-specific knockout or knockdown animals and the analysis of their phenotypes.

Initial approaches to identify p63 targets used vectors to overexpress TA- or ΔN-p63 isoforms in cultured cell lines. Ectopic expression of *TAp63* in Ptk2, HR9, F9 cell lines, or in lung epithelial cells induced *K14* expression [76, 77]. In contrast ΔN isoforms appear to have an inhibitory role in keratinocyte differentiation, while also inducing *K14* expression [64, 78]. These studies have clear limits: they cannot monitor cell-cell (epithelial-mesenchymal) interactions, tissue organization and stratification, but they can only reveal the expression of marker genes. More informative approaches have been employed to identify true TA- and ΔN-p63-specific targets by directly examining keratinocyte progenitor cells, with the added possibility to compare normal keratinocyte progenitors with ones derived from EEC patients or from animal models with *p63* mutations. Compared to studying the skin as a tissue (i.e., *in vivo* or in organ-type cultures), this approach is more likely to yield results relevant for skin development/maturation, as compared to using tumour or immortalized cells. A combination of isoform-specific siRNA-mediated downregulation in primary keratinocytes and *in vivo*, coupled to analysis in knockout and disease-specific knockin mice, has led to identify key target genes required for epidermal morphogenesis that are involved in pathogenesis of *p63*-linked ED [11, 54, 55, 79, 80]. The

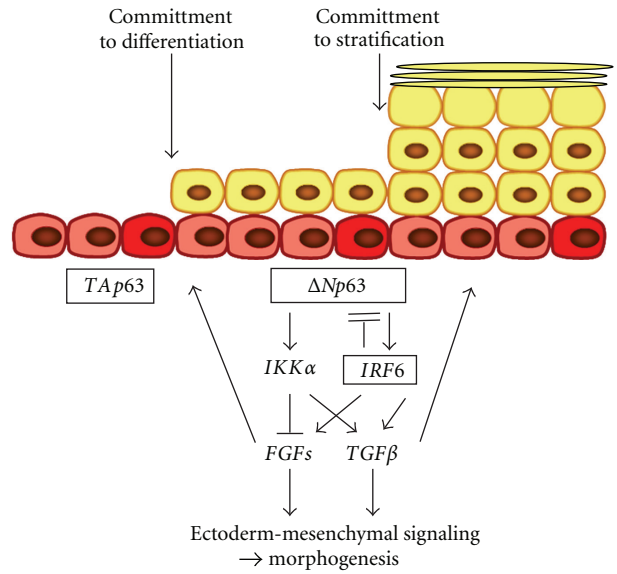

Figure 1: Schematic representation of known and proposed *p63* pathways relevant for epidermal differentiation, ectoderm-mesoderm signaling, and the onset of ectodermal dysplasias. *p63*, *IKKα*, and *IRF6* play a role in the developing epidermis, in a cell autonomous way to facilitate cell cycle arrest and differentiation. The same molecules also regulate the expression of soluble factors such as *TGFβ* and members of the *FGF* family to regulate the development of the underlying mesoderm, via epithelial-mesenchymal signaling. Disease genes are framed in boxes; regulations/interactions are indicated with lines or arrows.

gene coding for the protein kinase IKKα is a transcriptional target of ΔNp63, and indeed ΔNp63 mutant proteins found in EEC are unable to activate *IKKα* expression. IKKα is a component of the IkB kinase complex and is required for correct epidermal development and epithelial-mesenchymal interaction during development. Recent literature suggests a link between p63 and NFkB [81]; however, the kinase activity of IKKα is not required for its developmental function [40].

IKKα null mice display defects in epidermal, limb, and craniofacial development that are fully reverted after the reexpression of *IKKα* in the developing ectoderm [40]. Interestingly, IKKα was recently found to be a component of the TGFβ pathway in keratinocytes [82, 83] and to repress *FGF8* expression [40]. These observations link ΔNp63 function to the control of developmental signals (TGFβ and FGF8) regulating epidermal, limb, and craniofacial development (see Figure 1).

Similarly, *IRF6*, another ΔNp63 target gene, is involved in both epidermal development and limb/craniofacial development [55, 84]. The underlying mechanism relates to the ability of IRF6 to modify the stability of the ΔNp63 protein. *IRF6* expression is required at the onset of terminal differentiation to allow proteasome-dependent degradation of ΔNp63. Mutations of *IRF6* cause syndromes characterized by CL/P and other developmental anomalies, and *IRF6* knockin mice carrying the same mutation found in VWL patients (next paragraph) display a hyperproliferative epidermis that is unable to terminally differentiate [85].

In summary, IKKα and IRF6 are ΔNp63 targets and represent members of a regulated network that control stratification and terminal differentiation of the developing epidermis and also essential in the control of signalling from ectodermal cells to the mesoderm for the correct development of craniofacial structures and limbs. Finally, members of the BMP/TGFβ and FGF families should be regarded as important soluble mediators of this complex regulatory network [84].

6. The Role of *p63* in Palate Development

CL/P is a common feature in p63-linked syndromes that is thought to be related to alterations of a tightly controlled balance between proliferation and differentiation of precursor cells during ectoderm development [24]. Since p63 is a transcription factor, the basis of these defects logically resides in the inability of mutated p63 proteins to properly activate/repress the expression of target genes.

We [55] and others [84] have recently discovered that *IRF6* is a direct p63 target gene needed for palate development and skin differentiation. *IRF6* is a member of a family of interferon-dependent transcription factors [86] that control the proliferation-differentiation switch in epidermal cells [85, 87, 88]. *IRF6* is also required for palate closure: mutations in *IRF6* are linked to a set of syndromes related to ectodermal dysplasia [89], such as the Van der Woude syndrome (VWS, MIM 119300), an autosomal dominant disorder characterized by CL/P and dental anomalies, and the popliteal pterygium syndrome (MIM 119500) which, in addition to an orofacial phenotype similar to VWS, also displays syndactyly and ankyloblepharon [87, 90]. VWS is the most common CL/P syndrome, accounting for approximately 2% of all cases.

Importantly, the *p63* null, the *p63-R279H*, and the *Irf6-R84C* homozygous mice all display severe CL/P [55, 84, 85]. The phenotype observed in *Irf6* null mice is due to the inability of *Irf6* mutant cells to exit the cell cycle, leading to an undifferentiated hyperplastic skin [85, 87]. How this phenotype is linked to p63 is not totally clear; interestingly we noted that ΔNp63 is upregulated in the epidermis of *Irf6* null mice. Based on these findings, we propose the existence of a feedback regulatory loop between Irf6 and p63, in which p63 controls Irf6 transcriptionally, while Irf6 controls p63 at the protein-stability level. Importantly, these findings provide a molecular link connecting the *IRF6*- and *p63*-linked syndromes [55].

7. *p63*, Ectoderm Cell Layering and the Control of Limb Development

p63, *Dlx5;Dlx6*, and *FGF8* are coexpressed in the Apical Ectodermal Ridge (AER) of the mouse embryonic limb buds [35] as well as in the fins of the zebrafish embryos [6, 60]. The AER is a transitory ectodermal structure that rims the distal tip of the developing limbs, at the dorsal-ventral border, and is strictly required for limb bud expansion and morphogenesis, in particular for distal outgrowth and digit formation [91–93]. Recent models propose that signaling from the AER controls the generation of mesenchymal progenitors, in an instructive way [61, 94, 95]. The best characterized signals emanating from the AER cells are the FGFs, primarily FGF8, which is considered the key AER-derived instructive molecules driving proximo-distal morphogenesis of the limbs [41, 94, 96]. A complex network of reciprocal regulations between Wnt, FGF, and BMPs appears to be at the basis of the coordinated growth of the limb, resulting in time- and location-specific induction of subpopulation of skeletogenic progenitors [61, 91, 97, 98].

The AER is perhaps the first attempt of the embryonic (nonneural) ectoderm to organize into a multilayered epithelial tissue [93]. Considering that p63 has been shown to be required for epidermal stratification [10, 11, 99], it is not surprising that the AER of *p63* null or *p63 R279H* homozygous mice fails to organize as multilayer, in fact in the absence of functional p63 AER formation is nearly prevented. There is experimental evidence to indicate that a failure to maintain the AER is the main pathogenic mechanism in the onset of the ectrodactyly phenotype [93, 100]. In the *p63*, the *Dlx5;Dlx6,* and the *Dac* mutant mice (to name a few), the AER fails to express *FGF8* and appears poorly stratified. Thus, loss of AER stratification and reduced *FGF8* expression, whether induced by *p63* mutation or other genetic conditions, appear to be a common theme during the onset of this specific class of malformations. When *FGFR2* gene is deleted in the AER cells, via conditional genetics, the AER loses stratification and *FGF8* expression. In this case, the AER cells cannot respond to (AER-derived?) FGFs [94], that are apparently needed for AER maintenance. This is a strong indication that FGF signaling is needed by the AER cells to maintain stratification and function, apparently in an autocrine loop. It is important to note that loss of *FGF8* expression alone is not sufficient to explain the loss of AER stratification; however, we should consider that the reduced *FGF8* expression seen in SHFM is accompanied by altered expression of several other genes, that may exacerbate the suffering of the AER cells and result in reduced layering.

How is AER stratification controlled by p63? The activity of p63 is increasingly being linked to transcriptional regulation of molecules involved in cell adhesion [101]. Indeed, p63 has been shown to (directly or indirectly) control the expression of *EGF-receptor, ICAM, β4-integrin*, and *Claudin1* [3, 102–105]. Furthermore, the p53 responsive transmembrane protein *Perp* has been implicated in cell-cell adhesion and in the maintenance of epithelial integrity. *Perp*, a p63 target gene, is also positively regulated by both TA- and ΔN-p63 isoforms, and *Perp* null mice exhibit blistered stratified epithelium, likely due to compromised adhesion [106]. Recently *P-cadherin* (*CDH3* in human) has been shown to be a transcriptional target of p63 [37], and *P-cadherin* is the disease gene for the EEM syndrome [26, 27]. However, no limb developmental functions of P-cadherin have been recognized in mice [28], and therefore we currently lack a suitable animal model.

The link between altered adhesion properties and loss of stratification is not totally clear, although the capacity of ectodermal cells to form a multilayered epithelium certainly requires specific adhesionfunctions, distinct from

the single-layer naïve ectoderm. A "cadherin switch" model has been proposed to explain how cell-cell contact may potentially facilitate cell movement and layering [107]. It will be interesting to define how changes in p63 affect this network in a global way.

8. Do *p63*-Linked Pathways Converge on *p63* to Cause Limb Anomalies?

Expression of *FGF8* is strongly reduced in the AER of the *p63* null, *R279H p63* mutant, and *Dlx5;Dlx6* mutant embryos [33, 34], as well as several other mouse models of limb defects. A number of observations suggest that the p63 and the Dlx proteins may regulate *FGF8* expression by acting directly on the genomic region corresponding to the SHFM-III critical region [37, 108]: indeed true p63-binding sites are present within the region, as demonstrated by ChIP-seq screening [36], and several predicted *Dlx* binding sites cluster around the *FGF8* locus, in genomic regions conserved across mammalian species (see Supplementary Figures 1(a) and 1(b) available online at doi:10.1155/2011/864904). Assuming that *Dactylin* is not the disease gene for SHFM-III, then *FGF8* and components of the *NFkB* pathway might be the ones. It is tempting to speculate that the complex duplication-rearrangement modifies the position/organization of *cis*-acting control elements, which in turn may affects expression of *FGF8* and components of the *NFkB* pathway. Thus, in several ectrodactyly mouse models, downregulation of *FGF8* appears to be a common feature.

Another regulator of *FGF8* expression is the transcription factor Sp8. Animals null for *Sp8* show severe limb defects affecting the distal portion of the limbs, associated with a strongly reduced expression of *FGF8* [109–111]. *Sp8* is coexpressed with *Dlx* genes in the murine AER and forebrain [112] and appears in the top 1% of a list of conserved/coexpressed genes in microarray data (the CLOE algorithm [113, 114]; Dlx binding sites are also predicted in conserved regions near the *Sp8* locus (Supplementary Figure 1(c)). Therefore, *Sp8* is likely to be a *Dlx* target and to modulate *FGF8* expression.

Another pathway that controls *FGF8* expression links p63 with *IKKα*, a target of p63 relevant for ectoderm development and limb morphogenesis [54, 83, 115]. Interestingly, while mutations of *p63* and loss of *Dlx5;Dlx6* lead to a reduced *FGF8* expression in the AER, *IKKα* mutant embryos, the AER shows an increase of *FGF8* expression [40], a condition that nevertheless results in distal limb truncations and severe malformations.

From the above considerations, it appears that numerous players in the p63 network contribute to regulate *FGF8* expression in the AER. FGF8 is dynamically required for the correct establishment of signaling loops within the limb bud [61]—hence for normal limb development. Alterations in *FGF8* expression are closely linked to the onset of limb defects and malformations in several mouse models. The severity and extent of the malformation depends much on the time of onset of the AER deficit and the extent of (global) FGF reduction (including FGF4, −9, and −17), as indicated by recently reported results using mice with progressive and combined loss of single and multiple *FGF* genes [95]. Reduced *FGF8* expression and signaling is also closely linked to conditions that alter the AER morphology (reduced layering or altered adhesion). It is important to note that the AER of the AER-specific *FGF8* knock-out mice seems morphologically normal [38, 41, 116, 117]. We should also note, however, that in SHFMs the reduced *FGF8* expression is always accompanied by altered expression of several other genes.

The possibility that FGF8 is a common target of p63 network during limb development is in agreement with the well-known functions of FGF8 to sustain epithelial-mesenchymal signalling and assure the timely generation of the correct population of mesenchymal progenitors [95]. Should this hypothesis be true, this knowledge could be used in preclinical studies on mouse models, to attempt to restore sufficient levels of FGF exogenously, and hope to rescue normal limb development.

9. Dynamic Gene Expression of Partially Redundant Genes Is Critical for the Activity of *p63* and the Onset of Disease Phenotypes

An emerging theme in developmental biology is the importance of gene dosage and dynamic gene expression for correct morphogenesis [98]. Notable examples of this are the signaling functions of FGFs expressed in the AER [95], the gene-dosage-dependent functions of *Msx1* and *Msx2* for osteogenic differentiation of cranial neural crest cells [118], the progressive limb phenotypes and altered *Epha3* expression associated with the loss of posterior *HoxD* alleles [119], and the craniofacial phenotypes of embryos mutant for the Edn1-Dlx pathway [120]. Recent studies nicely illustrate the concept of "relativity of gene function", in particular during limb development [91]. In fact, early or late gene inactivation or misexpression can profoundly change the phenotypic outcome.

Consequently, such dynamic changes in gene functions may escape detection when genetic analysis is limited to constitutively null allele. On the same note, the function of individual genes is best examined upon partial and cumulative gene losses, and within the context of expression of related genes, as for example has been done in [95].

Several *Dlx* (*1, 2, 3, 5*, and *6*) and *FGF* (*4, 8, 9*, and *17*), genes are coexpressed in the AER, and their expression is dynamically regulated, both in respect with time (embryonic age) and location (territory of expression). In addition, there is evidence that *Dlx* and *FGF* genes are functionally redundant, at least in part. For example, no limb phenotype is observed in mice null for only one *Dlx* gene, while an ectrodactyly phenotype is observed in *Dlx2*;*Dlx5* null mice [121]. Furthermore, the ectrodactyly seen in the *Dlx5;Dlx6* mutant mice is fully rescued by the reexpression of only *Dlx5* [34]. An increased severity of craniofacial phenotypes correlates with progressive loss of more than one *Dlx* gene [120, 122]. Likewise, FGF8 and FGF4 represent the principal supply of FGF from the AER [38, 41], and FGF4 could functionally replace FGF8 [123]. All these are indication of a gene-dosage effect between functionally redundant genes.

We propose that the portion of the p63 network that (direct or indirect) regulates *FGF8* expression is exerted in a quantitative and dynamic mode. To support this, we should consider that although *p63* null and *p63EEC* homozygous mice show severe limb truncation or absence, the heterozygous mice appear to be normal. When heterozygous EEC mice are crossed with heterozygous *Dlx5;Dlx6* ones (also with apparently normal limbs), clear anomalies are observed, although mild [35].

A gene-dosage effect combined with the coexpression of functionally redundant genes implies the existence of a threshold level to be maintained to assure AER stratification and signaling functions. Following this logic, we have determined the expression of *Dlx* genes along the antero-posterior AER, by qPCR, and have noted that the expression of *Dlx2* and *Dlx5* is lower in the central portion of the AER, compared to the anterior or posterior segments. This may explain why in the absence of *Dlx5;Dlx6* only the central ray of the limb bud is affected: the central AER might be more sensitive to reduced *Dlx* expression due to intrisnic lower expression, and the loss of two or more *Dlx* gene may drop the level of pan-Dlx below the threshold.

On the same line, there is evidence that a certain amount of AER-derived pan-FGF is required to induce and maintain the underlying mesenchymal progenitors [61, 93, 95]. In fact, in the *Dlx5*;*Dlx6* DKO limbs, the reduction of *FGF8* expression is restricted to the central AER, the region where epithelial-mesenchymal signaling is primarily defective and the region where morphogenesis fails [33, 34]. Thus, the entire p63-Dlx-FGF is sensitive to gene dosage, timing, and position.

On the same note, we have determined the relative expression of *Dlx* genes comparing the FL and the HL at the same embryonic age, by qPCR. The results indicate that in the HL expression is generally lower (−15–20%) than that in the FL (Figure 3), supporting the relevance of dynamic expression for developmental defects. This may explain the finding that the HLs are usually more severely affected than the FLs. It is well known that the initiation and growth of the HL lags behind that of the FL [93]. Similarly, when *FGFR2* is conditionally deleted in the AER, the HLs are less severely affected then the FLs. In these mice, the expression of *FGF8* is first lost in the central wedge of the limb bud and this is associated with loss of stratification [94]. Thus, different dynamics of gene expression result in distinct phenotypic outcomes.

10. Emerging *p63* Regulatory Pathways—A Summary

Classical strategies have been adopted to search for phenotype-relevant p63 targets by "candidate gene" approaches or by genome-wide screenings. More modern approaches include the elaboration of a Position Weight Matrix combined with promoter occupancy data. It is expected that in the near future a wealth of high-throughput data (expressed genes and microRNA profiles, ChIP-seq, histone modification map) will be collected and new opportunities will emerge from meta-analyses of these complex data. In this direction, recently published work [36] reports the ChIP-seq data for p63 obtained from cultured keratinocytes. We should soon be able to define an evidence-based p63-network and thus raise novel hypotheses towards the identification of converging pathways and key players amenable to interventions. Here we summarize regulatory links that have either been demonstrated or appear highly likely and the supporting evidence (Figure 2).

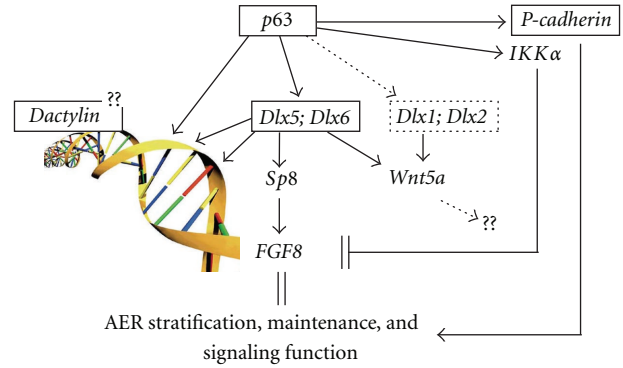

Figure 2: Schematic representation of known and proposed *p63* pathways relevant for limb development and the onset of ectrodactyly. *P-cadherin* is a known disease gene only in human, while *Dlx5;Dlx6* are known disease genes only in mice. *Dactylin* has been proposed as the disease gene for SHFM-III but no evidence for this is currently available. The most likely disease gene is *FGF8* (see text), regulated by both *p63* and *Dlx* proteins by *cis*- and *trans*-acting binding elements. The expression of *FGF8* and the stratification/maintenance/function of the AER are mutually essential (indicated by a double arrow). Disease genes are framed in boxes (solid for known or highly probable disease genes, dashed for putative ones); regulations are indicated with lines or arrows (solid for known or highly probable regulations, dashed for putative ones).

10.1. p63 Upstream of Ikkα. *Ikka* is a transcriptional target of p63 [54, 83, 115]. *Ikkα* null mice show malformations of the limb skeleton and abnormal epidermal differentiation. Surprisingly, in the absence of *Ikkα* the expression of *FGF8* is upregulated, indicating that any variation in *FGF8* level is deleterious for the ectoderm-derived cells [40].

10.2. p63 Upstream of Dlx5;Dlx6. *Dlx5* and *Dlx6* are coexpressed with p63 in the AER, and their expression is diminished in the absence of *p63* or in the presence of the *R279H* homozygous mutation [35]. This regulation occurs by (1) binding on the *Dlx5* and *Dlx6* promoters and activation of their transcription [35] and (2) distant regulation by an enhancer-like element located 250 kbp upstream of the *Dlx6* promoter [36]. Importantly, such p63-responsive enhancer element is deleted in one SHFM patient [36]. Also the expression of *Dlx1;Dlx2* is diminished in *p63* mutant embryonic limbs, and *Dlx1* and *Dlx2* are located near the critical region for SHFM-V. If the deletion alters their expression by positional effect and distal enhancers, *Dlx1;Dlx2* may turn out to be the SHFM-V disease genes.

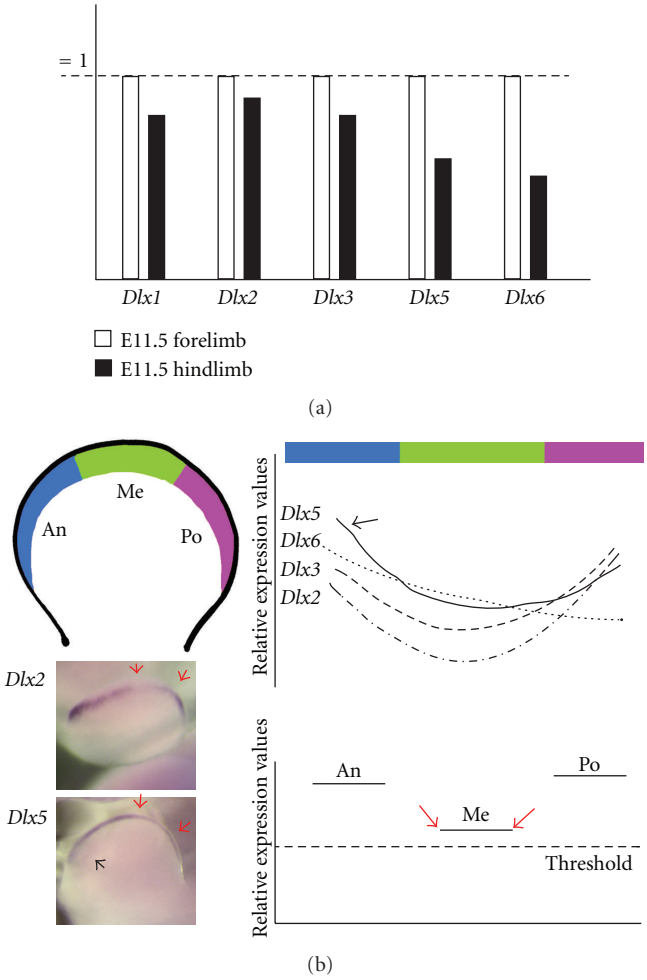

FIGURE 3: Quantitative and dynamic expression of *Dlx* genes during limb development. (a) Expression of *Dlx* genes in the hindlimbs (HL) and forelimbs (FL) limbs, at the same embryonic age, by qPCR. In the HL, the expression is always lower, as compared to the FL. This difference, most likely reflecting the developmental lag of the HL compared to the FL, may explain the finding that malformations affecting the HL are more severe. (b) Relative expression of *Dlx* genes along the length of the AER, from anterior (An) to medial (Me) to posterior (Po) regions (indicated with a color code). The expression of single *Dlx* genes is not uniform, and considering their partial redundancy the "global" expression is lower in the central wedge. This region of the AER appears to be more "sensitive" to genetic alterations and is specifically affected in SHFM.

10.3. p63 and Dlx5;Dlx6 Upstream of FGF8. Based on Expressed Gene profiling comparing p63-silenced keratinocytes with control ones [55] (A.C. unpublished), and based on recently published ChIP-seq data that identify true p63 binding sites within the SHFM-III critical region [36], there is a justified possibility that p63 regulates *FGF8* expression via *cis*-acting elements. Indeed, *FGF8* expression is strongly reduced in the AER of *p63* null and *p63 R279H* mutant embryos. *FGF8* expression is also diminished in the AER of *Dlx5;Dlx6* mutant embryos [33, 34]. Using a Position Weight Matrix approach to scan conserved regions of the mammalian genome for predicted Dlx binding sites, a high number of conserved Dlx-binding sites is found around the *FGF8* locus and in the SHFM-III critical region (Supplementary Figure 1).

10.4. p63 Upstream of Dlx3. *Dlx3* codes for a homeodomain transcription factor, member of the *distalless* family, expressed in keratinocytes [52] and responsible for p63 degradation during their differentiation [69, 70]. A negative feedback regulation between p63 and Dlx3 has recently been uncovered, that seems to be responsible for the TDO syndrome [124].

10.5. p63 Upstream of P-Cadherin. The *P-cadherin* gene (*CDH3* in human) has been shown to be a transcriptional target of p63 [27]. Interestingly, *P-cadherin* is mutated in the EEM syndrome, characterized by the presence of the SHFM phenotype [26, 27]. However, no limb developmental functions of P-cadherin have been shown in mice [28], and therefore the role of this molecule remains unclear.

10.6. Dlx5;Dlx6 Upstream of Wnt5a. Several observations suggest that Wnt5a is likely to be a transcriptional target of Dlx genes. First, *Wnt5a* expression is reduced in the AER of *Dlx5;Dlx6* mutant limbs (G.M. and LoIacono, unpublished), and Dlx2 and Dlx5 directly bind to regulatory elements in the *Wnt5a* locus and modulate transcription of *Wnt5a* in neural progenitors [125]. Second, *Wnt5a* null embryos exhibit a severe limb phenotype characterized by truncations of proximal elements and absence of the fingers, although the AER appears normal and expresses *FGF8* [126]. Third, *D-Wnt5* is a target of *distalles* in the *Drosophila* limbs [127]. Wnt5a is a short-range signaling molecule that could participate in a network of epithelial-mesenchymal signaling together with FGFs to induce and maintain specific pools of mesenchymal progenitors, as recently proposed [97]. One interesting possibility is that the activity of the "noncanonical" Wnt5a might antagonize the activity of "canonical" Wnts [128], known to be required for AER maintenance and limb morphogenesis [129–132].

11. Conclusions and Perspectives

Isoform-specific p63 transcriptional networks are being defined that begin to reveal the molecular basis for the dual function of this protein: (a) maintain the stem state of epidermal progenitors and (b) assure that cells can escape the stem state, exit the cell cycle, stratify and terminally differentiate. In the embryonic ectoderm, these apparently conflicting activities are strongly biased towards the maintenance of the stem state, while during subsequent development a finely-tuned equilibrium needs to be established to sustain turnover, regeneration, and differentiation. In EEC patients, the presence of one mutated *p63* allele generates a condition in which this equilibrium is compromised, and an altered progenitor-maintenance function ensues and results in a dysplastic skin. In order to further comprehend the role of p63 at the tissue level, mice null for *p63* appear not to be the ideal

model, as both the progenitor maintenance and the cell cycle escape functions are simultaneously compromised due to the total absence of the protein. Better animal models are therefore needed, such as isoform-specific knock-outs or knock-in of point mutated alleles. As today, the *EEC* and the *AEC p63* mouse models appear to better recapitulate the human diseases and should therefore be used more extensively.

The Apical Ectodermal Ridge (AER) is a region of the embryonic ectoderm specialized in signaling functions and responsible for outgrowth and patterning of the limbs. This function is exerted mostly via FGF signaling. The AER is perhaps the earliest attempt of ectodermal cells to organize into a multilayered tissue. Considering that p63 has been directly implicated in keratinocyte stratification, it is not surprising that the limb defects seen in EEC and related disorders consistently show loss of AER stratification, associated with reduced *FGF8* expression. Here we raise and justify the hypothesis that FGF8 might be the converging molecules on which various limb morphogenetic pathways centered on p63 impinge, directly or indirectly. We also present and discuss those indications suggesting that the AER-expressed *FGF*s and the *Dlx* genes act in a dose-dependent fashion and that their expression is dynamically regulated. Such "quantitative" effects have an influence on the establishment of correct signalling within the developing limb and could explain some features of the limb anomalies.

Recently published findings and unpublished results point to the importance of degradation of ΔNp63 during epidermis differentiation. This critical function appears to be exerted by at least three molecules (Dlx3, IRF6, Itch), although additional p63 degradation pathways are likely to emerge. In the near future, all aspects of the p63 upstream and downstream networks will be further clarified and initial efforts in this direction suggest the existence of an intricate network of regulations [133, 134].

Deciphering and comprehending the complete p63 network underlying cellular-developmental defects affecting ectoderm derivatives is the next critical task, expected to yield relevant new knowledge on the components of this network and on their regulation. Transcription profiling done on homogeneous cell populations and well-designed comparisons, have certainly been useful. However, for adult tissues or embryonic structures this approach is problematic and not easy to apply; furthermore, there are no cultured cell lines that can faithfully reproduce cell layering, "quantitative" gene expression, and dynamic signaling as they occur in the intact skin, palatal shelf, or limb bud.

Thus, scientist will engage in scientific activities that combine phenotype-causing targeted mutations followed by reexpression of a candidate target *in vivo* to monitor aggravation or correction of the developmental phenotype. In the case of the p63-Dlx regulation, this approach has been informative, and other best-candidates for being p63 and Dlx5;Dlx6 targets should likewise be tested. The *in vivo* approach is preferable, although time consuming, since p63 and most of the upstream and downstream players are express in a tissue-specific way and/or they are involved or depend on tissue interactions and signalling, notoriously difficult to be recapitulated *in vitro*. Emerging targets are IRF6, IKKα (for the epidermis, palate, and limbs), Wnt5a, and FGF8 (for the limbs), and important new discoveries are expected in this direction.

The long-term hope is to identify key enzymes or a ligand/receptor signaling systems, to be used to test the activity of exogenously provided ligands or active compounds in preclinical experimental setting, perhaps on skin, limb or palate organ-type cultures, and hope to revert the dysplastic/malformation phenotypes.

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